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PRINCIPAL INVESTIGATOR: Kenneth M. Noll

INSTITUTION: University of Connecticut

GRANT TITLE: Genetic Methods for Rapid Gene Localization in Hyperthermophilic Eubacteria

REPORTING PERIOD: 1 January 1991 - 31 August 1993 (32 months)

AWARD PERIOD: 1 January 1991 - 31 August 1993

OBJECTIVE: To develop genetic tools to investigate the molecular mechanisms of thermophily in and the evolution of the extremely thermophilic bacterium *Thermotoga neapolitana*.

APPROACH: We initiated investigations along several lines to develop genetic methods of analyses with *Thermotoga neapolitana*. (1) We investigated the conditions necessary for optimal growth of *T. neapolitana* on solid media under anaerobic conditions. (2) We attempted to isolate mutants to provide genetic markers for the development of gene transfer methods. (3) We explored a number of methods to introduce DNA into *T. neapolitana*. (4) A cryptic *Thermotoga* plasmid was discovered and its potential as a cloning vector was investigated. (5) A cosmid library and large chromosomal fragments will be used to located and map genes.

ACCOMPLISHMENTS (last 32 months): (1) We have tried a number of methods to cultivate *T. neapolitana* on solid media under strictly anaerobic conditions. Different types of polycarbonate anaerobic jars were tried and, although each performed well for up to a year, they all eventually failed to maintain anaerobiosis. We are currently using glass jars for routine cultures. (2) We isolated fifteen auxotrophic mutants after screening approximately 1,200 colonies resulting from EMS-mutagenized cells. Four required histidine (one of which reverted at a high frequency), one required leucine, one required adenine, and one required tryptophan.. The other auxotrophs require unknown factor(s) present in yeast extract and/or casamino acids. We have also isolated mutants resistant to elevated levels of 5-fluorouracil, 5-methyltryptophan, 6-azauracil, and 4-fluorophenylalanine. We encountered unexpected difficulties in our attempts to isolate *lac*⁻ mutants using X-gal. Some of our observations can be explained by the fact that two β -galactosidase genes may be present in *Thermotoga* (J. Moore, UCLA, unpublished results). (3) Using the various mutants we obtained, we investigated conjugation and transformation (both natural and electroporation) as means to introduce DNA into *T. neapolitana*. Although we had initial indications that this species is naturally competent for DNA uptake, subsequent work has been unable to verify this. The lack of good markers and vectors has been a major hindrance. (4) We discovered a cryptic miniplasmid in *Thermotoga* strain RQ7. The plasmid is 846 base pairs and encodes one basic protein of 25.5 kDa. A putative origin of replication has been identified and transcription/translation features observed. We are now trying to introduce genetic markers into the plasmid which might be useful for selections or screens in *T. neapolitana*. (5) Overlapping chromosomal restriction fragments have been identified using pulsed-field gel electrophoresis. A cosmid library is under construction and this will be used to locate and map genes.

SIGNIFICANCE: The proposed project is designed to develop the genetic tools

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necessary to increase our understanding of both thermophilic biological processes and the evolution of prokaryotes. Thermophiles are of interest for both their impact on the marine environment and their potential application to industrial processes. In this project we began the development of techniques that to readily identify and clone genes encoding proteins of interest. Phenotypic features of *T. neapolitana* that are of biotechnological interest include its ability to grow to high cell densities without producing sulfide (thus simplifying large-scale cultivation) and its unique, thermophilic degradative enzymes (proteases and carbohydrases). As a member of a phylogenetically deep bacterial lineage, it is of special interest for comparative studies with both bacteria and those archaea with which it shares physiological features.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS CITING THIS SUPPORT:

1. Harriott, O. T., R. Huber, K. O. Stetter, P. W. Betts, and K. M. Noll. A cryptic miniplasmid from the hyperthermophilic bacterium *Thermotoga* strain RQ7. (Submitted to *J. Bacteriol.*)
2. Vargas, M. and K. M. Noll. Selection of analog-resistant mutants of *Thermotoga neapolitana*. (manuscript in preparation)
3. Noll, K. M. "Developing Genetics with *Thermotoga*". Annual meeting of the American Society for Microbiology, May 1993. Atlanta, GA.
4. Childers, S. E., M. Vargas, and K. M. Noll. 1992. Improved methods for cultivation of the extremely thermophilic bacterium *Thermotoga neapolitana*. *Appl. Environ. Microbiol.* **58**:3949-3953.
5. Noll, K. M., M. Vargas, and S. E. Childers. 1992. Growth and selection of analog-resistant mutants of *Thermotoga neapolitana*. *Thermophiles: Science and Technology*. Reykjavik, Iceland, p. 99.
6. Vargas, M. and K. M. Noll. 1992. Isolation of analog-resistant mutants of the extreme thermophile *Thermotoga neapolitana*. NEMPET Annual Meeting. Blue Mountain Lake, N. Y.
7. K. M. Noll. 1992. The Biology of *Thermotoga neapolitana*. NEMPET Annual Meeting. Blue Mountain Lake, N. Y.

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Thermotoga neapolitana

- Maximal growth temperature 90°C
- Strict anaerobe; ferments sugars to hydrogen, acetate, lactate, and carbon dioxide; reduction of elemental sulfur to sulfide enhances growth
- Member of a slowly-evolving, deep phylogenetic lineage of bacteria
- Promising for genetic studies due to its aerotolerance, short generation time, and ability to grow on defined media

ACCOMPLISHMENTS

- Growth media and conditions defined
- Auxotrophic and analog-resistant mutants isolated
- Genetic transfer methods explored
- Miniplasmid discovered and characterized
- Chromosome map and clone library started

Genetic Methods for Rapid Gene Localization in Hyperthermophilic Eubacteria
OBJECTIVES

- Develop a method to introduce cloned DNA into *T. neapolitana*
- Isolate mutants conferring selectable phenotypes
- Screen other *Thermotoga* strains for plasmids for use as cloning vectors
- Construct a genome map from large fragments of the chromosome

SIGNIFICANCE

- Develop techniques to readily identify and clone protein-encoding genes
- Develop *T. neapolitana* as a source of thermophilic degradative enzymes
- Study biogenic sulfide production in marine geothermal areas
- Study the evolution of marine thermophiles